# A DIAGNOSTIC DIPSTICK FOR ROTAVIRUS IN FAECES. THE USE OF COLLOIDAL SELENIUM AS MARKER

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## SUMMARY

Rotavirus is known to be the most common cause of severe diarrhea in infants and young children, each year leading to an estimated 800 000-900 000 deaths. Rotavirus also infects bovines and other species, with high morbidity and mortality.

We have developed a fast and simple **naked-eye** dipstick system to detect human and bovine rotavirus in faeces, using nitrocellulose as solid phase, two monoclonal antibodies, and colloidal selenium as marker. The system is able to detect $10^4$ viral particles (v.p.; 1-2 ng)/g of faeces.

When compared with a commercial latex system, the specificity and sensitivity of the test for human rotavirus were 100%. The test was superior than an ELISA prepared with the same monoclonal antibodies. For bovine rotavirus the specificity and sensitivity of the test were also 100%, in comparison with RNA-polyacrylamide gel electrophoresis.

## RESUMEN

Los rotavirus son el agente causal más frecuente de diarreas severas en los niños y jóvenes, dejando cada año un estimado de alrededor de 800 000-900 000 muertes. Infecta también a otras especies, como la bovina, con una alta morbimortalidad.

Nosotros hemos desarrollado un sistema diagnóstico rápido y simple de lectura visual, que detecta rotavirus humano y bovino en heces fecales. El sistema emplea nitrocelulosa en la fase sólida, dos anticuerpos monoclonales y selenio coloidal como marcador, y es capaz de detectar $10^4$ partículas virales(p.v., 1-2 ng)/g de heces.

Para rotavirus humano la especificidad y la sensibilidad del sistema fueron del 100%, al compararse con un látex comercial, y mayor que para un ELISA basado en los mismos anticuerpos monoclonales. Para la detección de rotavirus bovino la especificidad y la sensibilidad del sistema fueron del 100%, comparado con la electroforesis de ARN en gel de poliacrilamida.

## INTRODUCTION

Rotavirus (RV) is now known to be the most common cause of acute gastroenteritis in children (about 30-60% of the total), and a major cause of infant mortality in developing countries, each year accounting for an estimated 800 000-900 000 deaths world-wide (Blacklow *et al.*, 1991). RV also represents an important economic problem for breeders of cattle, pigs and others species (Durigon *et al.*, 1991; Frías, 1983).

While RNA-polyacrylamide gel electrophoresis (RNA-PAGE, Frías *et al.*, 1984) is still used for the detection of RV, automated (i.e. microELISA) and, more recently, fast and simple diagnostic systems (i.e. latex agglutination), have gained widespread acceptance (Westmorland *et al.*, 1987; Kohli *et al.*, 1989; Durigon et al., 1991; Giordano *et al.*, 1991). A majority of these tests are based on poly- and monoclonal antibodies (Kohler and Milstein 1975).

In this paper we present a simple, 30-minute, **naked-eye**, nitrocellulose (NC) dipstick diagnostic system for human and bovine RV in faeces. The system, that employs two MAbs, and colloidal selenium (coSe) as non-metallic marker, detects $10^4$ viral particles (1-2 ng) per g of faeces.

## MATERIALS AND METHODS

### Antigens and standard

The human RV strains Wa (serotype 1), DS-1 (serotype 2) and Hochi (serotype 4), were provided by Center for Biological Research of Havana. The bovine strain RF and the simian strain SA-11 were supplied by National Center for Agriculture & Cattle Health (CENSA). All strains were multiplied in MA-104 kidney epithelial cells, as suggested by Sato *et al.* (1982) for 72 h, until a cytopathogenic effect was observed in more than 90% of the cells. The sensitivity of the diagnostic system was determined using a RV laboratory standard composed by DS-1 purified virus with a titer of $10^7$ virus particles/ml.

### Monoclonal antibodies

The mouse MAbs CB-R.1 and CB-R.2 were used in the experiments. Both MAbs are IgG1k and recognize different epitopes of the 41 kDa specie-specific antigen VP6 (Araña *et al.*,

1986). The MAbs were purified from ascitic fluid by affinity chromatography on Protein A-Sepharose CL 4B (Pharmacia; Ostlund, 1986).

## Conjugation of antibodies with colloidal selenium

CoSe-antibody conjugates were prepared as previously described by van Doorn *et al.* (1989).

Briefly, the optimal pH and the minimal amount (MPA) of MAb needed to protect the coSe sol from flocculation in the presence of salts were determined. For this, serial dilutions of MAbs in water, at different pH, were made. Adsorption of MAbs to coSe sol was performed at room temperature, and a 1:10 (v:v) ratio during 1 h, under gentle agitation. One volume of 10% sodium chloride (Merck, FRG) was added to ten volumes of adsorbed coSe, for 5 min and flocculation was read at 540 nm, in comparison with 0% flocculation (no sodium chloride), and 100% flocculation (sol without MAb and with 10% sodium chloride).

After determining optimal pH and MPA, the adsorption of MAbs to the coSe sol was scaled-up to the required volumes. The conjugates were blocked with 0.2% casein (Sigma, USA), for 30 min. Free MAbs and casein were eliminated by centrifugation at 10 000 rpm, during 30 min. The pellet was suspended in 20 mM anhydrous dipotassium hydrogen phosphate (Merck, FRG), 0.5% bovine serum albumin (BDH), 0.05% sodium azide (BDH), at pH 8.5, and stored at 4°C.

## Specimens

Faeces from 86 infants and children up to 5 years old, that arrived to the Center for Hygiene and Epidemiology of Havana (CHEH) with acute diarrhea, were used in our experiments. Detection of bovine RV was performed in 56 samples from calves with acute gastroenteritis.

Specimens were diluted 1:4 (v:v) in a sample dilution buffer, composed of phosphate buffer saline (PBS), pH 7.4, and 1% BSA, mixer in a Vortex apparatus until dissolution and centrifuged at 3000 rpm, during 10 min. The supernatants were employed as samples in the tests.

## Nitrocellulose coating and washing conditions

NC strips (0.2 nm, Schleicher & Schuell) were sensitized by spotting 1 $\mu$l of MAbs, at different concentrations, in a 5 mM sodium chloride buffer, pH 8.0. The strips were blocked with PBS-2% BSA, 1 h at 37°C, washed with PBS, and air-dried. When incubated with test samples, the sensitized strips were washed 3 times with PBS-0.05% Tween-20.

## RV detection techniques

Polyacrylamide gel electrophoresis for viral RNA (RNA-PAGE) was performed as previously described by Frías *et al.* (1984), and taken as reference method for bovine RV.

For the detection of human RV, the commercial **Diarlex-Rota** kit (Orion Diagnostica) was taken as reference procedure, and used according to the manufacturer's instructions. A laboratory **sandwich** microELISA system that employs CB-R.1 and CB-R.2 (Araña *et al,*. 1986) was also used for comparisons. Briefly, the plate is coated with 10 $\mu$g/ml of CB-R.1 of 150 mM $Na_2CO_3/NaHCO_3$, pH 9.6 and 200 $\mu$l/well overnight, at 4°C. The plate is washed twice with 0.05% Tween-20 and 200 $\mu$ l of diluted samples and controls are incubated during 2 h at 37°C. The plate is washed 6 times as before and 200 $\mu$l of working dilution of conjugate CB-R.2-Peroxidase is added to each well and incubated for another 1 h at room temperature. The plate is washed 10 times again and 200 $\mu$l of the sustrate is added, in this case a 1,2 phenylendiamin is used in the proportion of 5 mg per 10 ml of 0.1 M citric acid/$Na_2HPO_4$, pH 5.0 and 5 $\mu$l of 30% $H_2O_2$. The plate is incubated at room temperature during 30 min and the reaction is stoped with 2.5 M $H_2SO_4$, 50 $\mu$l/well. The results can be read at 492 nm or visually.

Discordant samples were analyzed by transmission electron microscopy (TEM), using negative staining with 2% phosphotungstic acid (Balzers) during 10 min. Samples were observed in JEOL-JEM 2000 EX Microscope, with 80 kV and x80 K magnification.

## Sensitivity and specificity

All assays were made in triplicate. The following mathematical expressions were used to calculate the sensitivity and specificity of the dipstick method:

$$S = \frac{CPS}{PS} \times 100\%$$

$$Sp = \frac{CNS}{NS} \times 100\%$$

The Positive and Negative Predictive Values (PPV, NPV) were calculated as follows:

$$PPV = \frac{CPS}{TPS} \times 100\%$$

$$NPV = \frac{CNS}{TNS} \times 100\%$$

where: **S** = Sensitivity, **Sp** = Specificity, **CPS** = No. of Coincidence Positive Samplex, by both the Dipstick Test and the Reference Test, **PS** = No. of Positive Samplex by the Reference Test, **CNS** = No. of Coincidence Negative Sampplex by both the Dipstick Test and the Reference Test, **NS** = No. of Negative Samplex by the Reference Test, **TPS** = Total of Positive Samplex by the Dipstick Test, **TNS** = Total of Negative Samplex by the Dipstick Test.

# RESULTS

## Development of a diagnostic dipstick for rv

CB-R.2 was selected to be conjugated to coSe because of its lower MPA with respect to CB-R.1 (80 $\mu$g/ml of MAb of sol = 1 MPA, vs. 160 $\mu$g/ml = 1 MPA, at pH 8.0).

To select the best coating conditions, different concentrations of CB-R.1 were spotted onto the NC. The strips were incubated with one ml of positive sample for 20 min, washed, and further

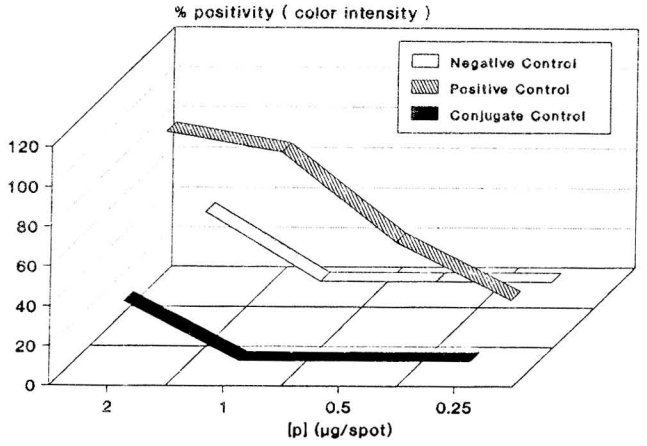


**Fig.1.**- Determination of optimal NC coating conditions. Positive control = DS-1 standard, 100 ng/ml; negative control = PBS.

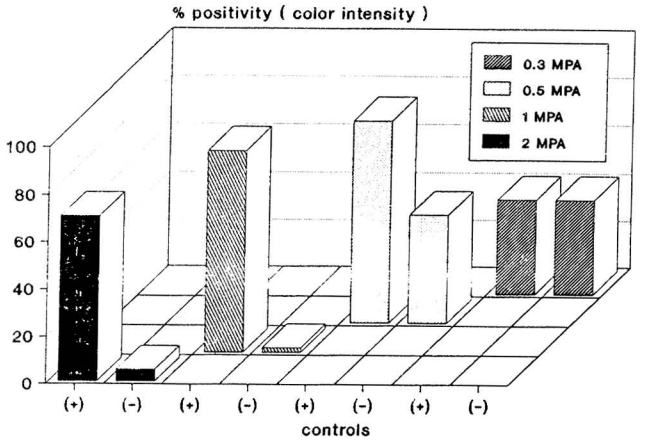


**Fig. 2.** Determination of optimal conjugate conditions. (+) = positive control, DS-1 standard, 100 ng/ml; (-) = negative control, PBS.

incubated with 1 ml of the 1 MPA coSe conjugate of CB-R.2, for 10 min. All procedures were developed at room temperature and with gentle agitation. As shown in figure 1, 1 $\mu$g of CB-R.1 per spot provided the highest specific color signal.

In order to minimize the total amount of MAb used per determination, several dilutions of the 1 MPA CB-R.2 coSe conjugate were evaluated, using the CB-R.1 coating conditions mentioned above. It can be seen from figure 2 that with a 1:1 (v:v) dilution of 1 MPA (40 $\mu$g/ml) satisfactory results were obtained.

Once the best MPA and coating concentration were determined, the whole procedure was carried out in one single step, by incubating strip, sample and conjugate together during 30 min, without washing. This method showed less sensitivity than the two-step procedure (results not shown), and was abandoned.

## Specificity and sensitivity

All human and bovine RV strains were identified with the dipstick system (figure 3). The sensitivity level was determined using the RV laboratory standard as been approximately $10^4$ viral particles (1-2 ng) per gram of faeces/ml (figure 4).

For human RV, the specificity and sensitivity of the dipstick system were 100%, when compared with the Diarlex-Rota commercial latex kit (Table 1). The ELISA showed a lower sensitivity in our studies (93.3%), as 8 discordant ELISA-negative samples were found positive by TEM.

For bovine RV detection, the specificity and sensitivity of the dipstick were also 100%, when compared with traditional RNA-PAGE (Table 2).

**Table 1**
Comparison of the DIPSTICK system with LATEX and ELISA for human RV detection

| | LATEX | | | ELISA | | |
|---|---|---|---|---|---|---|
| Dipstick | Positive | Negative | Total | Positive | Negative | Total |
| Positive | 1 | 0 | 1 | 14 | 8* | 22 |
| Negative | 0 | 19 | 19 | 1 | 63 | 64 |
| Total | 1 | 19 | 20 | 15 | 71 | 86 |
| Sensitivity | | | 100% | | | 93.3% |
| Specificity | | | 100% | | | 100% |
| PPV | | | 100% | | | 100% |
| NPV | | | 100% | | | 98.4% |

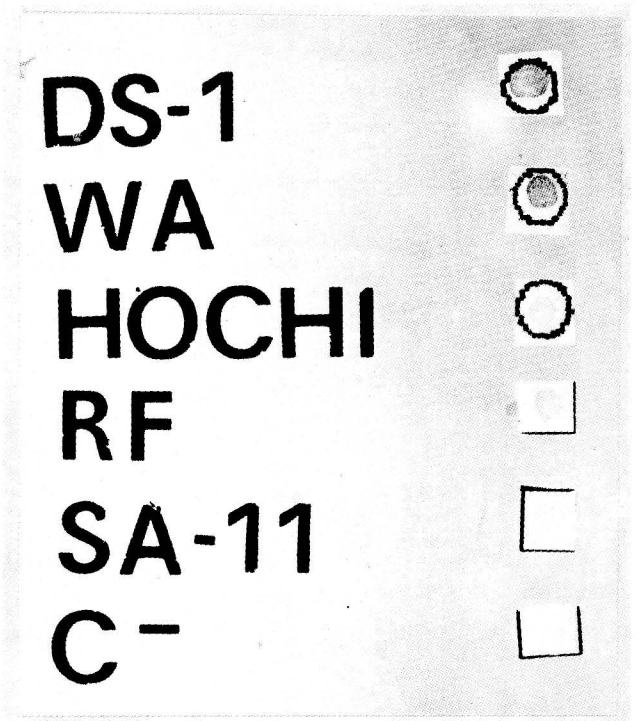


**Fig. 3.** RV dipstick recognition pattern for different strains. CB-R.1: 1 $\mu$g/ml; CB-R.2-CoSe conjugate: 1 MPA (1:1 dilution); negative control = non-infected cell supernatant.

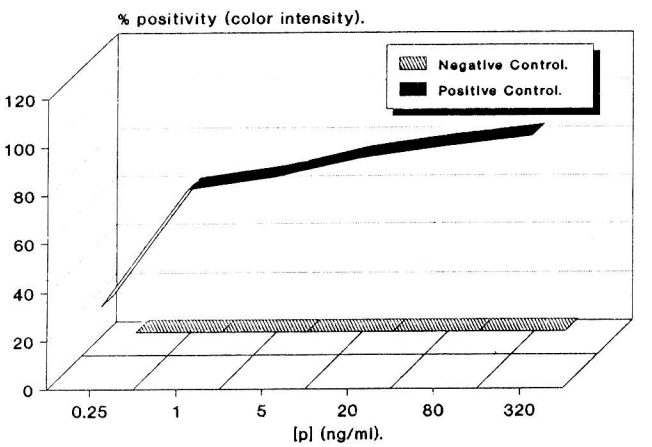


**Fig. 4.**- Sensitivity limit determination of the RV diagnostic dipstick system. CB-R.1: 1 $\mu$g/spot; CB-R.2-CoSe conjugate:1 MPA, (1:1 dilution); positive control = DS-1 standard; negative control = PBS.

## DISCUSSION

Colloidal markers, based on gold (De Mey and Moeremans 1983; Shanfun *et al.*, 1991) or selenium (van Doorn et al., 1989) can be advantageous over enzymatic markers in immunodetection techniques, as: (*a*) washings and substrate steps are not required and total time is reduced, (*b*) long-lasting visual results are produced, (*c*) no costly equipment is needed, (*d*) the reagents maintain adequate stability at 4°C, and (*e*) all incubations can be carried out at room temperature.

Our dipstick diagnostic system for RV is based on an antigen-antibody reaction occurring in a NC strip, visualized by coSe agglutination. A specific MAb is coated onto the NC, made to react with the test sample, and the strip incubated with a second specific MAb, conjugated with the coSe sol. A positive reaction, indicating that RV has been **sandwiched** between both antibodies, is visualized as an orange spot in the NC. The color intensity depends of the positivity of the sample; negative samples develop no color.

In our experience, this is the first report of the use of non-metallic coSe as marker for RV diagnosis. Our simple and fast dipstick test (30 min with precoated strips) proved to be reproducible, versatile (human and bovine RV), and with a sensitivity level in the same range of other available diagnostic systems, as RNA-PAGE, ELISA, and latex. The system components have maintained their stability for more than half year at 4°C (results not shown).

The dipstick system can be used in small laboratories, doctor's offices, emergency rooms, or in field conditions, eliminating the requirement of sample transportation and/or accumulation. At present, we are evaluating the possibility of preparing the samples by manual agitation and filtration, in order to

**Table 2**
Comparison of the DIPSTICK system with RNA-PAGE for bovine detection.

| | RNA-PAGE | | |
|---|---|---|---|
| Dipstick | Positive | Negative | Total |
| Positive | 23 | 0 | 23 |
| Negative | 0 | 33 | 33 |
| Total | 23 | 33 | 56 |
| Sensitivity | | | 100% |
| Specificity | | | 100% |
| PPV | | | 100% |
| NPV | | | 100% |

eliminate the centrifugal step, and making the system more attractive for field conditions, where equipment is scarce.

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